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Synergistic Antibiofilm Potentials of Essential Oil of Clove (Eugenia Caryophyllata) and Nisin Against Bacillus Cereus Biofilms

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ABSTRACT

Bacillus cereus is an important foodborne pathogen that can contaminate a wide range of foods and form persistent biofilms on food and food-contact surfaces, thereby increasing the risk of spoilage and cross-contamination. In response to growing interest in natural antimicrobial agents for food preservation, this study investigated the antimicrobial and antibiofilm activities of nisin and clove essential oil against B. cereus, with particular emphasis on their potential synergistic effects when used in combination. Antimicrobial activity was evaluated using agar diffusion and broth tube dilution assays, while biofilm formation and inhibition were assessed using Congo red agar and crystal violet microtiter plate assays. The results showed that nisin exhibited strong antimicrobial activity against B. cereus, whereas clove essential oil alone showed little inhibitory effect on bacterial growth under the tested conditions. However, clove essential oil enhanced the antimicrobial activity of nisin when used in combination. In the antibiofilm assay, nisin alone showed strong activity, with all tested concentrations inhibiting biofilm formation by more than 90%. Clove essential oil alone also reduced biofilm formation, with inhibition ranging from 68.5% to higher levels depending on concentration. The combination treatments produced the greatest antibiofilm effects, with the most effective treatment, 250 IU/mL nisin combined with 1500 IU/mL clove essential oil, achieving 98.6% inhibition. Overall, these findings suggest that although clove essential oil alone was ineffective against planktonic growth, it contributed substantially to enhanced antimicrobial and antibiofilm activity when combined with nisin. The combination of nisin and clove essential oil may therefore represent a promising natural strategy for controlling B. cereus and its biofilm in food-related environments.

KEYWORDS

Bacillus cereus (B. cereus); Nisin; Eugenol; Clove essential oil (Syzygium aromaticum); Antimicrobial activity; Antibiofilm activity; Synergistic effect; Food safety

1. INTRODUCTION

Food safety remains a major global challenge, as foodborne pathogens continue to cause substantial illness, mortality, and food spoilage worldwide. Among these pathogens, Bacillus cereus is widely distributed in agricultural products and is an important cause of food contamination and spoilage, particularly in foods such as rice, pasta, dairy products, cereals, and spices [1-3]. In addition to its pathogenicity, B. cereus is capable of forming biofilms on food and food-contact surfaces, which increases its persistence and makes contamination more difficult to control. Spore formation during biofilm development may further enhance its survival in food-processing environments [4-7].

Natural antimicrobial compounds have attracted increasing attention as alternatives to conventional chemical preservatives. Among these, plant-derived substances, particularly essential oils, have been widely investigated for their antibacterial and antifungal activities [8-11]. Clove (Syzygium aromaticum), a spice belonging to the Myrtaceae family, has long been recognised for its preservative potential owing to its strong antimicrobial properties [12]. Clove essential oil is rich in phenolic compounds, especially eugenol, which has shown antimicrobial and antibiofilm activities against several foodborne bacteria [13, 14]. These effects are believed to involve disruption of cell membrane integrity, inhibition of protein synthesis, and interference with nucleic acid synthesis [15].

Nisin is a bacteriocin produced by certain strains of Lactococcus lactis subsp. lactis and has long been recognised as an effective antimicrobial agent in food systems. It is particularly active against Gram-positive bacteria and bacterial spores, and acts mainly by forming pores in the cell membrane and binding to lipid II, thereby inhibiting cell wall biosynthesis [16-18]. In addition to its direct antibacterial effect, the synergistic activity of nisin in combination with plant-derived compounds has been reported for decades. Studies since the early 2000s have consistently shown that nisin exhibits synergistic antimicrobial activity when combined with plant-derived compounds. Previous studies have also shown that nisin can act synergistically with plant-derived compounds, including thymol, garlic extract, carvacrol, eugenol, and cumin seed essential oil, against pathogens such as Listeria monocytogenes, Bacillus subtilis, and Bacillus cereus [19-23]. These findings suggest that combining nisin with clove essential oil may provide an effective strategy for controlling B. cereus growth and biofilm formation.

Based on this background, the present study aimed to evaluate the antimicrobial and antibiofilm activities of nisin and clove essential oil against Bacillus cereus, with particular emphasis on their potential synergistic effects when used in combination. As B. cereus is a significant foodborne pathogen capable of forming persistent biofilms on food and food-contact surfaces, identifying effective natural antimicrobial combinations may offer a practical alternative for food preservation and contamination control. It was hypothesised that the combination of nisin and clove essential oil would produce stronger inhibitory effects on bacterial growth and biofilm formation than either agent used alone.

2. METHODOLOGY

2.1. Materials and Preparation

2.1.1. Preparation of the Clove Oil Extract

A modified extraction procedure was used in this study to obtain a clove-derived extract essential oil. Conventional clove essential oil extraction is commonly performed by steam distillation, hydrodistillation, solvent extraction, or Soxhlet extraction [24]. Clove buds were obtained from a local supermarket in Birmingham, United Kingdom. Prior to extraction, the cloves were blended with anhydrous magnesium sulfate at a ratio of 25:1 (w/w) and pulverised into a fine powder. The powder was then combined with olive oil at a ratio of 1:1 (w/v) and incubated at 50°C for 30 min. After extraction, the mixture was filtered to remove insoluble material. The filtrate was further treated with methanol to wash away the carrier oil and residual impurities, and the resulting clove-derived extract was collected for antimicrobial and antibiofilm assays. The clove oil working solution was then serial diluted with 0.5 % acetone and were prepared and used immediately.

2.1.2. Preparation of Nisin

Nisin from L. lactis was purchased from Sigma-Aldrich (St. Louis, MO). A 2.5% (w/v) nisin solution was prepared in 0.05% hydrochloric acid (HCl), left to stand for 10 min, and sterilised by filtration through a 0.22 μm Millipore membrane filter.

2.1.3. Culture of the Bacillus Cereus

Bacillus cereus NC11143 used in this study was provided by the Biochemical Engineering Laboratory, University of Birmingham, UK. The strain was maintained on nutrient agar plates (Oxoid, CM0001, Basingstoke, UK) at 4°C. For culture preparation, B. cereus was inoculated into 100 mL of nutrient broth (Oxoid, CM0001, Basingstoke, UK) in a universal flask and incubated at 37°C for 18 h with shaking at 150 rpm. The standard inoculum was prepared based on the linear relationship between optical density (OD) and colony-forming units (CFU), which was established using culture-based enumeration and spectrophotometric measurements at 600 nm with a Jenway 6300 spectrophotometer (Bibby Scientific Ltd., Stone, UK). The standard inoculum utilised in this study was adjusted to 4 × 107 CFU/ml by phosphate buffered saline.

2.1.4. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis of the Clove Oil

The chemical composition of the clove oil was analysed by gas chromatography (GC-MS). A 5 µg/mL clove oil (CO) solution was prepared using acetone as the diluent. The analysis was conducted according to a previously reported method using a Shimadzu GC-2010 system [25]. Separation was carried out on a ZB-5 fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Phenomenex, USA). Helium was used as the carrier gas, and methanol was used as the blank. The injection volume was 2 µL. The initial oven temperature was set at 60 °C and held for 1 min, then increased to 120 °C and held for 2 min, followed by further increases to 210 °C and finally 250 °C. The final temperature of 250 °C was maintained for 30min

2.1.5. Detection of Biofilm Formation By Congo Red Agar Assay

The biofilm-forming ability of Bacillus cereus was assessed using the Congo red agar (CRA) method according to a previously reported procedure (Katongole et al., 2020). The CRA medium was prepared using 8 g/L Congo red dye (Oxoid, UK), 50 g/L sucrose (Oxoid, UK), and 37 g/L brain heart infusion agar (Oxoid, UK). Each component was autoclaved separately at 121°C for 15 min and then combined at 55°C before use. B. cereus was inoculated onto the CRA plates by streak plating and incubated at 37°C for 24–48 h. Following incubation, black colonies with a dry crystalline appearance were interpreted as positive for biofilm formation [26], whereas red or pink colonies were considered indicative of non-biofilm-forming isolates. As shown in Figure 1, the presence of black colonies indicated that B. cereus was capable of forming biofilm.

Figure 1. The Congo Red Agar plate with B. cereus after incubation

2.1.6. Agar Diffusion Assay

The agar diffusion assay, first described by Bauer et al. [27], was used to evaluate the susceptibility of B. cereus to nisin, clove essential oil, and their combinations. To assess the antimicrobial activity at different concentrations, nisin and clove essential oil were prepared separately at concentrations ranging from 125 to 1000 IU and 1500 to 6000 IU, respectively. Combinations of different concentrations of nisin and clove essential oil were also tested to evaluate their interaction effects.

The prepared nisin solution was diluted with sterile water, while clove essential oil was diluted with acetone. The B. cereus culture was diluted to 10-5 in PBS and spread onto Mueller–Hinton agar plates, which are widely used for routine antimicrobial susceptibility testing due to their high reproducibility, low levels of inhibitors, and suitability for the growth of most bacterial pathogens [28]. Wells were then made in the inoculated agar plates, and 0.1 mL of each antimicrobial preparation was added into each well. Tetracycline was used as the positive control. The plates were incubated upright at 37°C for 24 h. After incubation, the presence of a clear inhibition zone around the well indicated antimicrobial activity. The diameter of each inhibition zone was measured in millimetres (mm).

2.1.7. Determination of MIC (Tube Dilution Method)

The minimum inhibitory concentration (MIC) of nisin, clove essential oil, and their combinations against B. cereus was determined using the broth tube dilution method [29]. Nisin and clove essential oil were tested individually and in combination at concentrations ranging from 125 to 1000 IU and 1500 to 6000 IU, respectively. The bacterial culture was diluted to 10-5 in PBS, and each tube contained 1 mL Mueller–Hinton broth, 1 mL of antimicrobial solution, and 0.1 mL of diluted B. cereus culture. Tetracycline served as the positive control. Initial bacterial growth was assessed by plating onto nutrient agar and measuring OD at 600 nm. Following incubation at 37°C for 24 h, OD600 and viable growth on nutrient agar were assessed again. MIC was determined by comparing bacterial growth before and after incubation.

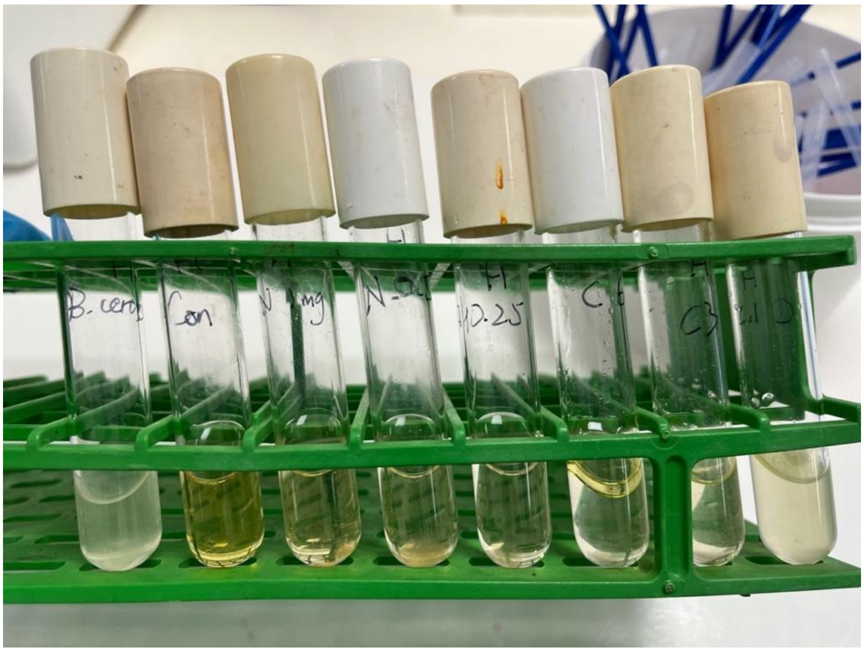

Figure 2. The tube dilution method. Different concentrations of nisin and clove essential oil were tested. As shown in the figure the difference in turbidity can be seen clearly after incubation

2.1.8. Biofilm Formation Assay

Biofilm formation was assessed in 96-well polystyrene microtitration plates according to a previously reported method [30]. For evaluation of B. cereus biofilm formation, the bacterial culture was first diluted to 10-5 in PBS. Then, 50 µL of the fresh standardised bacterial suspension and 50 µL of nutrient broth were added to each well. The microtitration plates were incubated at 37°C with shaking at 150 rpm for 48 h. After incubation, the contents of each well were gently discarded by tapping the plate, and the wells were washed with 200 µL sterile PBS to remove non-adherent cells. The attached biofilm was then stained with 200 µL of 0.1% (w/v) crystal violet and left at room temperature for 20 min. Excess stain was removed by washing the wells with sterile deionised water. The stained biofilm was subsequently fixed with 200 µL of 96% ethanol, and the absorbance of the adherent stained bacteria was measured at 600 nm using a microplate reader.

2.1.9. Antibiofilm effect determination

The antibiofilm effects of nisin, clove essential oil, and their combinations on B. cereus biofilm formation were evaluated using 96-well polystyrene microtitration plates. Nisin and clove essential oil were tested individually at concentrations ranging from 125 to 1000 IU and 1500 to 6000 IU, respectively, and their combinations were also examined. For each well, 50 µL of fresh standardised B. cereus culture, 50 µL of nutrient broth, and 50 µL of the antimicrobial preparation were added. The plates were then incubated at 37°C with shaking at 150 rpm for 48 h. After incubation, the contents of the wells were gently discarded by tapping the plates, and the wells were washed with 200 µL sterile PBS to remove non-adherent cells. The remaining biofilm was stained with 200 µL of 0.1% (w/v) crystal violet and left at room temperature for 20 min, followed by washing with sterile deionised water. The stained biofilm was then fixed with 200 µL of 96% ethanol. Control groups included a growth control (B. cereus + broth), media control (broth only), sterility control (broth + antimicrobial agent), and stain control (crystal violet only). The absorbance of the stained adherent cells was measured using a microplate reader, and the percentage of biofilm inhibition was calculated according to a previously reported method and formula [5].

$$\text{Percentage of Biofilm Inhibition} = [(\text{OD growth control} - \text{OD sample})/\text{OD growth control}] \times 100$$

2.2. Statistical Analysis

All experiments were replicated in a sterile environment. All data were analysed using GraphPad Prism 9 software and presented as mean ± standard deviation (SD). Differences between groups were analysed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. A p value of less than 0.05 was considered statistically significant. In the figures, statistical significance was indicated as $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

2.3. Ethical Consideration

Upon the commencement of this study, approval to conduct the study was obtained from the School of Chemical Engineering, University of Birmingham. The study consisted of experiments that involved the use of microbial cultures (Bacillus cereus) and numerous chemicals. Biological and chemical risk assessments were undertaken and authorised by the department to ensure the health and safety of the researcher and laboratory users.

3. RESULT AND DISCUSSION

3.1. GC-MS Analysis of Clove Oil Extract Components

Based on comparable maceration-type extraction methods reported in the literature, the expected yield of the clove-derived extract obtained by this modified oil-assisted extraction procedure may fall within the low single-digit range, approximately 3–8% (w/w) [31, 32], although the exact yield would depend on solvent recovery efficiency and post-extraction handling. According to the chemical composition of the clove oil extract analyzed by GC-MS, Eugenol and eugenyl acetate were identified as the dominating constituents, accounting for approximately 80% of the extract. Other detected components included β-caryophyllene, β-caryophyllene oxide, α-humulene, and δ-cadinene. These findings are consistent with previous reports [33, 34], which similarly identified eugenol as the predominant component of clove essential oil. Eugenol has also been widely reported to exhibit antimicrobial and antibiofilm activities against food-associated Gram-positive bacteria [35].

3.2. Antimicrobial Activity of Nisin and Clove Essential Oil Against Bacillus Cereus

3.2.1. Agar Diffusion Assay

The agar diffusion assay demonstrated that nisin exerted strong antimicrobial activity against Bacillus cereus, and the inhibition increased with concentration. According to Zhao et al. [36], antibacterial activity can be classified as weak (≤12 mm), moderate (12–20 mm), and strong (≥20 mm). In the present study, all tested concentrations of nisin produced inhibition zones greater than 20 mm, indicating strong antibacterial activity. This finding is consistent with previous studies reporting the effectiveness of nisin against B. cereus and other Bacillus spp. [37-39]. However, Mauricio et al. [40] reported only moderate inhibition against Bacillus spp. at comparable nisin concentrations, which may reflect differences in experimental conditions such as pH and incubation temperature. Since nisin is known to perform better under lower pH and temperature conditions [21], the absence of pH measurement in the present study may partly explain this inconsistency.

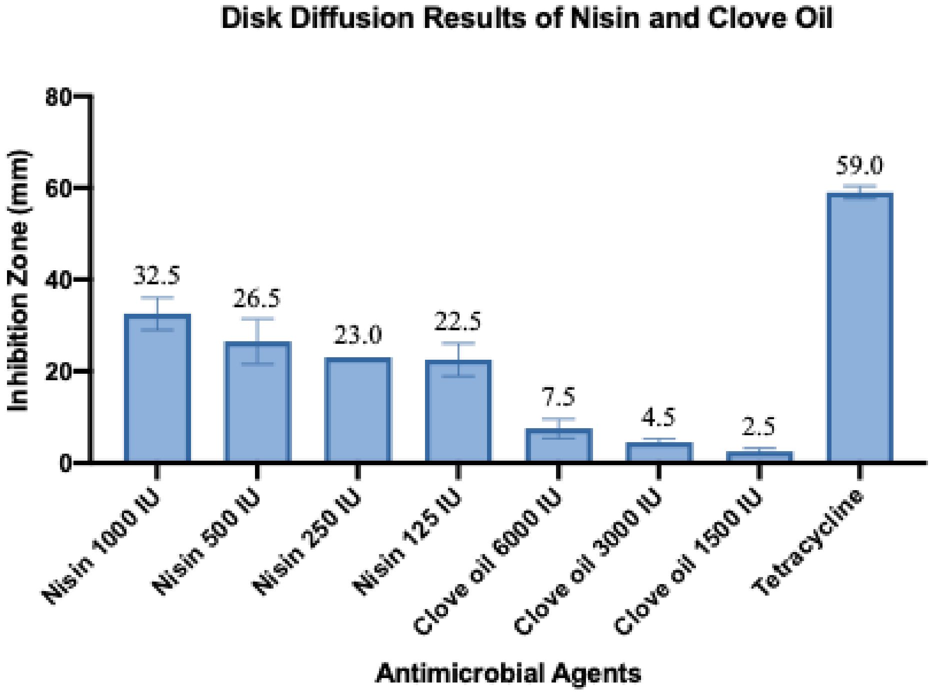

Figure 3. The inhibitory effects of nisin and clove essential oil against B. cereus at different concentrations between (125IU–1000IU), (1500IU-6000IU)

As illustrated in Figure 3, clove essential oil did not show effective antimicrobial activity against B. cereus under the tested conditions, very limited inhibition zone was observed at any concentration. This result differs from previous reports showing that clove essential oil inhibited Gram-positive bacteria, including S. aureus and B. cereus [41, 42]. Such discrepancies may be attributed to differences in assay conditions, particularly temperature and solvent system. Valero et al. [42] observed inhibition of B. cereus in carrot broth at 16°C, while Pajohi et al. [23] also suggested that the antimicrobial performance of essential oils may vary with temperature. In addition, the hydrophobic nature of essential oils may limit their diffusion in agar, which could further contribute to the absence of inhibition observed in this study.

Despite the absence of appreciable antimicrobial activity when clove oil was used alone, its combination with nisin markedly enhanced the inhibition of B. cereus. As shown in Figure 4, all combination treatments produced larger inhibition zones than clove oil alone, and these increases were statistically significant across all three clove oil concentrations tested. For clove oil at 3000 IU, all combinations with nisin resulted in significantly greater inhibition than clove oil alone ($p < 0.05$). A similar trend was observed for clove oil at 6000 IU, where combinations with 1000 and 500 IU nisin showed significant increases ($p < 0.01$), the combination with 250 IU nisin showed a smaller but still significant increase ($p < 0.05$), and the combination with 125 IU nisin produced the greatest inhibition zone overall (34 mm; $p < 0.001$). For clove oil at 1500 IU, all nisin combinations also exhibited significantly stronger inhibition than clove oil alone, with the combinations containing 1000, 500, and 250 IU nisin showing very strong significance ($p < 0.001$), while the 125 IU nisin combination remained significantly different ($p < 0.01$).

These findings indicate that clove oil, although ineffective when applied alone under the tested conditions, contributed to enhanced antimicrobial activity when combined with nisin. Most combination treatments produced inhibition zones above 20 mm, indicating strong antibacterial activity, although the magnitude of inhibition did not follow a fully linear concentration-dependent pattern. This suggests that the interaction between clove oil and nisin may not be explained solely by dose, but may instead reflect a more complex synergistic relationship between the two agents. Similar synergistic effects between nisin and plant-derived antimicrobials have been reported previously [21, 23, 36, 43]. Singh et al. [20] likewise showed that a plant extract with little antimicrobial activity on its own could still enhance the antibacterial activity of nisin when used in combination. Therefore, the present results support the view that clove oil can potentiate the antimicrobial effect of nisin against B. cereus, even though it shows little or no direct inhibitory activity when used alone.

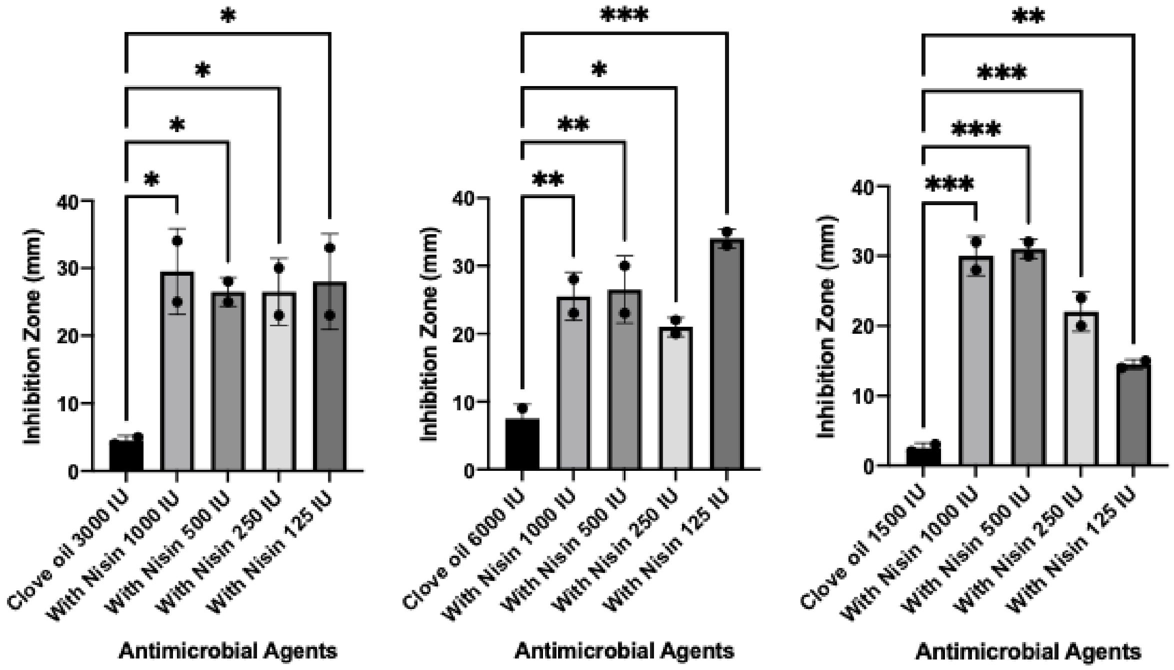

Figure 4. The inhibitory effects of clove oil in combination with nisin against Bacillus cereus as determined by agar diffusion assay

3.2.2. Tube Dilution Method

The broth tube dilution assay showed that nisin inhibited the growth of B. cereus in a concentration-dependent manner. At concentrations ranging from 250 to 1000 IU/mL, nisin effectively suppressed bacterial growth, while 1000 IU/mL showed the strongest inhibitory effect, with no colony formation observed before or after incubation. At 500 and 250 IU/mL, only a small number of colonies were present initially, but no colonies were detected after incubation, indicating effective inhibition. In contrast, 125 IU/mL nisin did not completely inhibit bacterial growth, as small numbers of colonies were still observed both before and after incubation. Although the OD value at 125 IU/mL decreased to some extent, it remained higher than that observed for the other nisin concentrations. According to the definition of MIC as the lowest concentration that prevents visible microbial growth after overnight incubation [44], the MIC of nisin against B. cereus in the present study was determined to be 250 IU/mL. This value is higher than that reported in some previous studies. For example, Kramer et al. [45] reported considerably lower MIC values for nisin against Gram-positive bacteria, while Rajkovic et al. [37] found that the MIC of nisin against B. cereus varied with both strain type and incubation temperature. Therefore, the relatively higher MIC observed in the present study may be associated with differences in bacterial strain and experimental temperature.

Consistent with the agar diffusion assay, clove essential oil alone did not exhibit a clear inhibitory effect against B. cereus. At 1500 and 3000 IU/mL, visible turbidity remained before and after incubation, indicating that these concentrations were insufficient to inhibit bacterial growth. Although some reduction in turbidity was noted, particularly at 1500 IU/mL, this did not correspond to complete inhibition. At 6000 IU/mL, clove essential oil also failed to suppress growth, as abundant colony formation and an increase in final OD value were observed after incubation. This result may be explained by the fact that the antimicrobial performance of essential oils is highly dependent on the test system and composition. Previous study reported that the composition of oils obtained by different extraction method may bring affections to antimicrobial properties [46]. In addition, as mentioned above, the antibacterial activity of essential oils against B. cereus can vary with temperature and growth conditions [42]. Therefore, the weak antibacterial effect of clove essential oil in the present study may be related to the assay conditions rather than a complete absence of intrinsic activity. In this context, the plate count results were considered more reliable than OD changes for interpretation of bacterial inhibition.

The combination treatments showed stronger antimicrobial effects than either clove essential oil alone or the corresponding concentrations of nisin alone, indicating a synergistic interaction. Except for the combinations containing 125 IU/mL nisin, most combination treatments showed almost no colony formation either before or after incubation. This suggests that the presence of clove essential oil enhanced the inhibitory activity of nisin against B. cereus. Similar synergistic effects have been reported previously for nisin combined with plant-derived compounds. Ettayebi et al. [19] demonstrated that thymol enhanced the antimicrobial activity of nisin against L. monocytogenes and B. subtilis, while Periago et al. [21] found that low concentrations of carvacrol, although ineffective alone, significantly increased the antibacterial effect of nisin against B. cereus. A similar pattern was observed in the present study. However, the combinations containing 125 IU/mL nisin were less effective than the other combination groups. In particular, the combinations of 125 IU/mL nisin with 1500 or 3000 IU/mL clove essential oil still showed small numbers of colonies before and after incubation, resembling the result of 125 IU/mL nisin alone. Among the 125 IU/mL combinations, only the treatment with 6000 IU/mL clove essential oil showed improved inhibition, as no colonies were detected after incubation, although this effect was still weaker than that observed for the other combination groups. As the OD values of some treatments did not consistently reflect the plate count results, plate counting appeared to provide a more reliable basis for interpretation. Based on colony formation, the combination of 125 IU/mL nisin and 6000 IU/mL clove essential oil was considered the minimum inhibitory concentration for the combined treatment.

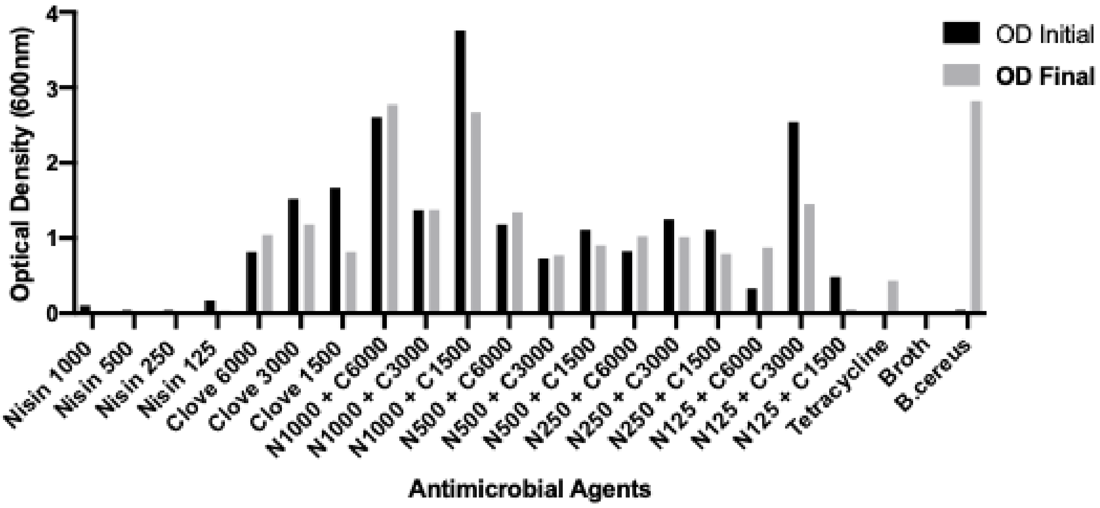

Figure 5. The OD value initial and final result of the tube dilution method with different concentrations of nisin, clove oil and their combinations (unit: IU/ml, N refers to Nisin, C refers to Clove oil)

3.3. Antibiofilm effect of nisin and clove essential oil

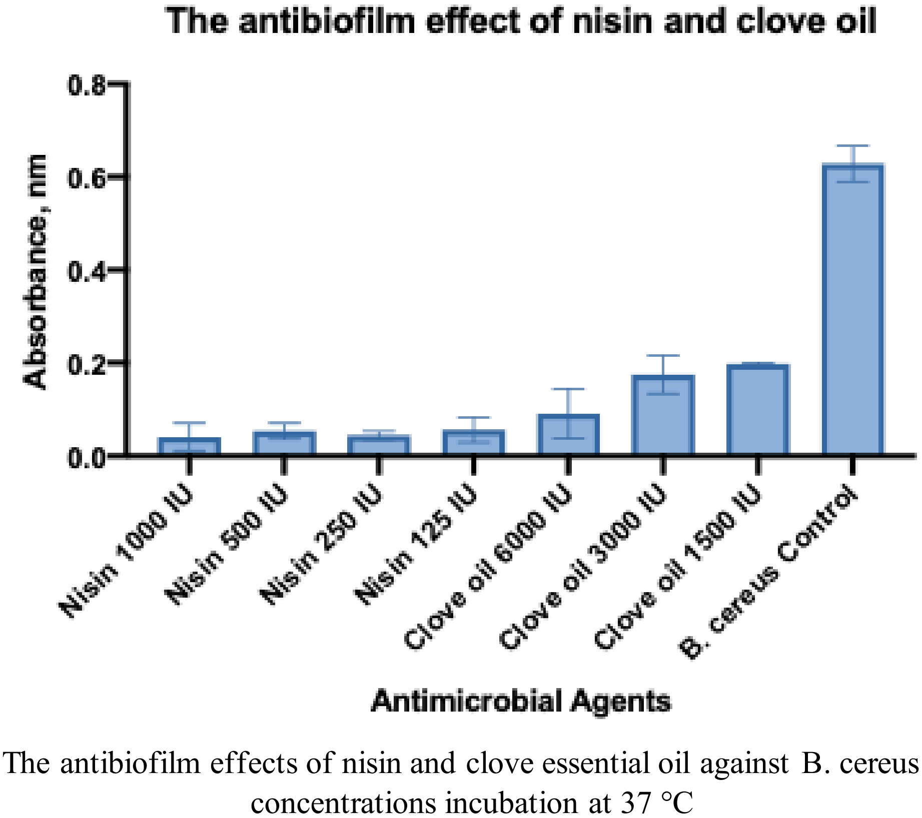

Figure 6. The antibiofilm effects of nisin and clove essential oil against B. cereus at different concentrations incubation at 37 °C

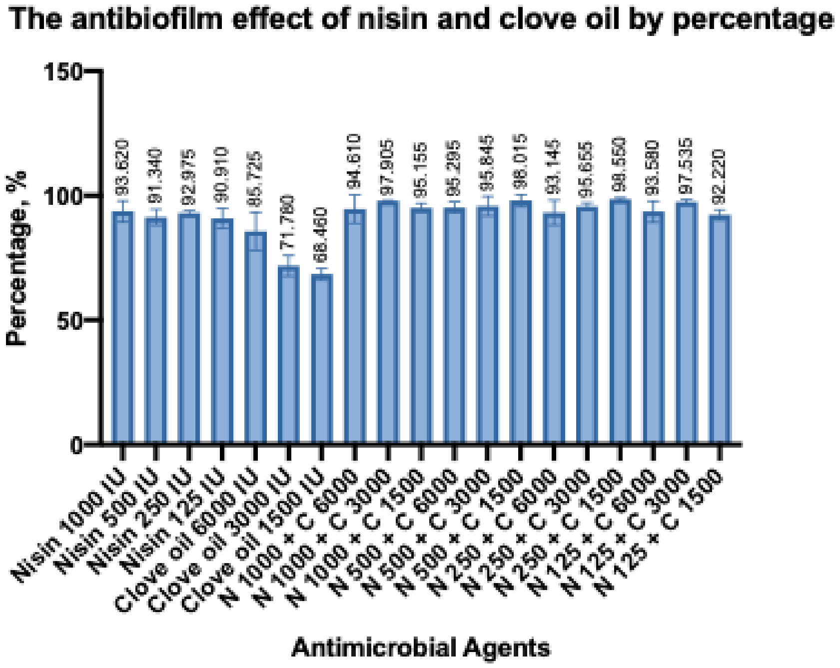

Figure 7. The percentage of anti-biofilm effects of nisin and clove essential oil against B. cereus at different concentrations incubation at 37 °C (N refers to Nisin, C refers to Clove oil).

Figure 6 illustrated the results of the crystal violet assay. It was confirmed that Bacillus cereus was able to form biofilm under the experimental conditions, as the untreated control showed an OD value of 0.63. As illustrated in figure 7, all tested treatments reduced biofilm formation to some extent, and all inhibition values were greater than 50%, indicating good antibiofilm activity according to the criterion proposed by Olawuwo et al [47]. Among all treatments, the combination of 500 IU/mL nisin and 1500 IU/mL clove essential oil showed the strongest antibiofilm effect, with an inhibition percentage of 98%, whereas clove essential oil at 1500 IU/mL used alone showed the lowest inhibition, at 68.5%.

Nisin alone exhibited strong antibiofilm activity against B. cereus at all tested concentrations. This finding is consistent with previous studies showing that nisin can inhibit biofilm formation in a range of foodborne bacteria, including Listeria monocytogenes, Staphylococcus aureus, Salmonella enteritidis, and Bacillus spp. [48, 49]. It also supports the conclusion that nisin is not only active against planktonic cells, but can also interfere with biofilm establishment. However, the magnitude of inhibition observed in the present study appears greater than that reported by Bag and Chattopadhyay [50], who found that nisin alone did not achieve more than 50% inhibition against B. cereus biofilms. This discrepancy may be related to differences in bacterial strain, incubation conditions, medium composition, and assay design.

Unlike the results of the agar diffusion and broth dilution assays, clove oil alone also showed clear antibiofilm activity in the microtiter plate assay. Although its effect was weaker than that of nisin and the combination treatments, all tested concentrations of clove essential oil inhibited biofilm formation to a substantial extent. This result is in agreement with previous reports showing that clove essential oil can inhibit biofilm formation in Gram-positive bacteria, including L. monocytogenes, S. aureus, and Bacillus subtilis [51-53]. The difference between its poor antimicrobial effect on planktonic growth and its relatively strong antibiofilm activity may suggest that clove essential oil acts more effectively on early adhesion or biofilm development than on actively growing planktonic cells. This may also be related to the activity of eugenol, the major active component of clove oil, which has been reported to reduce biofilm biomass even at sub-inhibitory concentrations [54].

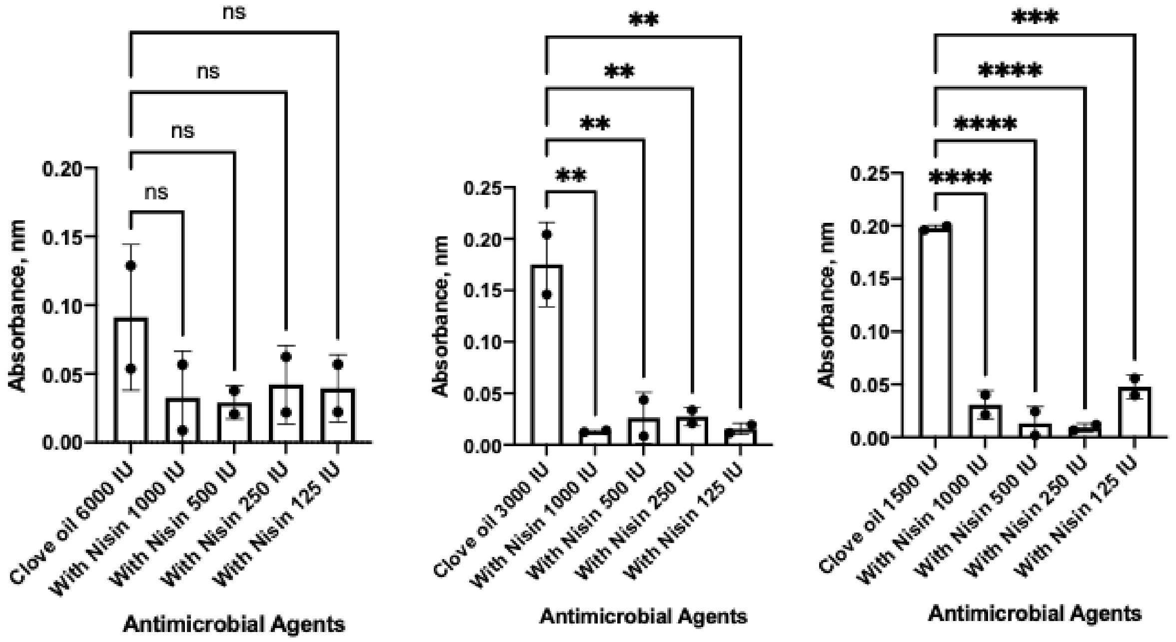

Figure 8. Antibiofilm effects of clove oil alone and in combination with nisin against Bacillus cereus

The combination treatments generally reduced biofilm biomass more effectively than clove oil alone, although the extent of this effect depended on the clove oil concentration. As shown in Figure 8, when clove oil was used at 6000 IU, all combinations with nisin produced lower absorbance values than clove oil alone, indicating reduced biofilm formation; however, these differences were not statistically significant (ns). In contrast, at 3000 IU clove oil, all combination treatments resulted in significantly lower absorbance than clove oil alone ($p < 0.01$), demonstrating an enhanced antibiofilm effect in the presence of nisin. A more pronounced pattern was observed at 1500 IU clove oil, where combinations with 1000, 500, and 250 IU nisin showed highly significant reductions in absorbance ($p < 0.0001$), while the combination with 125 IU nisin also remained significantly different ($p < 0.001$). These findings indicate that the antibiofilm activity of clove oil was strengthened by combination with nisin, particularly at the lower clove oil concentrations tested.

This pattern suggests that the interaction between nisin and clove oil was concentration dependent. The absence of significant differences at 6000 IU may indicate that clove oil alone had already exerted a relatively strong inhibitory effect on biofilm formation at this concentration, leaving less room for further enhancement by nisin. By comparison, at 1500 and 3000 IU, the addition of nisin substantially improved antibiofilm efficacy, supporting a potentiating effect between the two agents. This observation is broadly consistent with previous studies showing that nisin can enhance the antibiofilm activity of plant-derived compounds and essential oil constituents [43, 50].

3.4. Limitations and Further Research

Several limitations of this study should be acknowledged. First, the clove oil used in this study may not have possessed sufficient purity, which could have affected its observed antimicrobial and antibiofilm activities. Second, only a single strain of Bacillus cereus was investigated. Given that different strains may vary in their antimicrobial susceptibility, growth characteristics, and biofilm-forming capacity, the generalisability of the present findings to other B. cereus strains may be limited. Finally, all experiments were conducted under controlled laboratory conditions and did not include evaluation in real food matrices or on food-contact surfaces. Consequently, the practical applicability of nisin, clove oil, and their combinations in actual food-processing environments cannot be fully established from the present study and should be further examined in future work.

4. CONCLUSIONS

In conclusion, this study demonstrated that nisin showed strong antimicrobial activity against Bacillus cereus, whereas clove essential oil alone showed little or no antibacterial effect under the tested conditions. However, clove essential oil enhanced the activity of nisin when used in combination, indicating a synergistic antimicrobial effect. In the biofilm assay, both nisin and clove essential oil were able to inhibit biofilm formation, and the combination treatments produced the greatest antibiofilm activity. Overall, these findings suggest that while clove essential oil alone was ineffective against planktonic growth, its combination with nisin may offer a promising natural strategy for controlling B. cereus and its biofilm in food-related environments. Further studies should investigate the active components of clove oil and evaluate the combined effects of nisin and clove oil under different environmental and food-processing conditions. Their practical effectiveness on food-contact surfaces and in real food systems should also be assessed.

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